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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — UNITED KINGDOM

72.23522.001

4. Title of the invention — FUSION PROTEINS

5. Name of your agent *(if you have one)*

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FUSION PROTEINS

FIELD OF THE INVENTION

The present invention relates to a process for the activation of lymphocytes and the use of activated lymphocytes in the treatment of cancer. The invention also relates to fusion proteins for the activation of lymphocytes, to nucleic acids encoding the fusion proteins and to vectors carrying the nucleic acids.

BACKGROUND OF THE INVENTION

Lymphocytes require at least two distinct signals in order to respond to antigens by activation of effector functions (Bretscher and Cohn 1970 Science 169: 1042-1049; Crabtree 1989 Science 243: 355-361). The primary signal is specific for antigen. For B-lymphocytes, the B-cell antigen receptor (surface immunoglobulin) recognises three-dimensional epitopes on a variety of macromolecules. For T-lymphocytes, the T-cell receptor (TCR) recognises peptide antigens displayed on the surface of antigen-presenting cells by proteins of the major histocompatability (MHC) family (Weiss et al. 1986 Ann. Rev. Immunol. 4: 593-619). Stimulation of the primary signal in isolation normally leads to apoptosis (programmed cell death) of the lymphocyte or leads to the establishment of a state of sustained unresponsiveness or anergy (Weiss et al. *supra*). In order to achieve activation of the lymphocyte, accessory signals are required which may be delivered by cytokines or by cell-surface co-stimulatory ligands present on antigen-presenting cells (APC). There are a number of such co-stimulatory molecules now identified including adhesion molecules, LFA-3, ICAM-1, ICAM-2. Major co-stimulatory molecules present on APC are the members of the B7 family including B7-1 (CD80), B7-2 (CD86) and B7-3. These molecules are ligands of co-stimulatory receptors on lymphocytes including CD28 (WO92/00092), probably the most significant

co-stimulatory receptor for resting T-cells. Different members of the B7 family of glycoproteins may deliver subtly different signals to T-cells (Nunes et al. 1996 J. Biol. Chem. 271: 1591-1598).

Established tumours, despite the fact that they commonly express unusual antigens on their surfaces, are poorly immunogenic. It has been postulated previously that one method for stimulating immune recognition of tumour cells would be to enhance antigen presentation and co-stimulation of lymphocytes in the context of tumour antigens. Transfection of the genes encoding B7-1 and B7-2, alone or in combination with cytokines, have been shown to enhance the development of immunity to experimental tumours in animal models (e.g. Leong et al. 1997 Int. J. Cancer 71: 476-482; Zitvogel et al. 1996 Eur. J. Immunol. 26:1335-1341; Cayeux et al. 1997 J. Immunol 158:2834-2841). However, in translating these results into a practical treatment for human cancer, there are a number of significant problems to be overcome. A major problem in such studies is the need to deliver B7 genes *in vivo* to a large number of cells of the tumour to achieve efficacy. A second problem is that it is important to target expression of B7 to the tumour cells to avoid inappropriate immune cell activation directed against other cell types.

The present invention solves these problems by delivering a gene encoding a secreted co-stimulatory molecule (SCM) with binding affinity for a tumour antigen. In this way, a relatively small number of transfected cells within the tumour act as a local factory to produce the co-stimulatory molecule which is shed from the producer cell and binds to other cells in the tumour. The invention has the additional advantage that tumour cells need not be the target for transfection. There are a number of cell types present within the tumour cell mass in addition to the cancerous cells. These can include cells of the tumour vasculature (e.g. endothelial cells) and immune cells which infiltrate the tumour, such as tumour-infiltrating lymphocytes (TIL) and macrophages (Normann 1985 Cancer

Metastasis Re. 4:277-291; Leek et al 1996 Cancer Res. 56: 4625-4629). Any of these cell types can be targeted for expression of the SCM and can serve as a local factory within the tumour for production of SCM. Preferably, the cells used to produce the SCM are the cancerous cells, endothelial cells or macrophages. Alternatively, the progenitors of monocytes or endothelial cells may be targeted, such as CD34-positive peripheral blood mononuclear cells (Asahara et al. 1997 Science 275: 964-967).

The secreted co-stimulatory molecule of the invention (SCM) is a novel engineered fusion protein comprising a signal peptide for secretion from mammalian cells, at least one antigen-binding domain from an immunoglobulin or an immunoglobulin-like molecule and at least one further domain which acts as a co-stimulatory signal to a cell of the immune system. The use of combinations of SCMs containing different co-stimulatory domains is also envisaged. The SCMs are produced by expression of SCM-encoding genes in the autologous cells of the individual to be treated and hence any post-translational modifications added to the protein by the host cell are authentic and provide fully functional protein and appropriate pharmacokinetics.

WO 92/00092 describes truncated forms of B7-1, derived by placing a translation stop codon before the transmembrane domain, secreted from mammalian cells. In that particular case, a heterologous signal peptide from the Oncostatin M gene was used. WO 92/00092 also describes fusion proteins which contain the extracellular domain of B7-1 fused to the Fc region of an immunoglobulin. Such molecules can bind to CD28 on T-cells and serve to stimulate T-cell proliferation. However such stimulation occurs only to a moderate extent unless the B7 or B7-derivative is immobilised on a solid surface.

Gerstmayer et al. (1997 J. Immol. 158: 4584-4590) describes a fusion of B7-2 to an scFv specific for ErbB2 followed by a myc epitope

tag and polyhistidine tag which is secreted when expressed in the yeast *Pichia pastoris*. This molecule retained binding for antigen and co-stimulated proliferation of T-cells prestimulated with PMA and IL-2. However, glycosylation of such a molecule is of the yeast type, which is likely to lead to inappropriate pharmacokinetics in humans.

A large number of monoclonal antibodies and immunoglobulin-like molecules are known which bind specifically to antigens present on the surfaces of particular cell types such as tumour cells. Procedures for identifying, characterising, cloning and engineering these molecules are well established, for example using hybridomas derived from mice or transgenic mice, phage-display libraries or scFv libraries. Genes encoding immunoglobulins or immunoglobulin-like molecules can be expressed in a variety of heterologous expression systems. Large glycosylated proteins including immunoglobulins are efficiently secreted and assembled from eukaryotic cells, particularly mammalian cells. Small, non-glycosylated fragments such as Fab, Fv, or scFv fragments can be produced in functional form in mammalian cells or bacterial cells.

The immunoglobulin or immunoglobulin-like molecule may be derived from a human antibody or an engineered, humanised rodent antibody such as a CDR-grafted antibody or may be derived from a phage-display library or may be a synthetic immunoglobulin-like molecule.

The antigen-binding domain may be comprised of the heavy and light chains of an immunoglobulin, expressed from separate genes, or may use the light chain of an immunoglobulin and a truncated heavy chain to form a Fab or a $F(ab)'_2$ fragment. Alternatively, truncated forms of both heavy and light chains may be used which assemble to form a Fv fragment. An engineered scFv fragment may also be used, in which case, only a single gene is required to encode the antigen-binding domain. Preferably the antigen-binding domain is formed from a Fv or a scFv.

The co-stimulatory domains can be chosen from extracellular portions of the B7 family of cell-surface glycoproteins, including B7-1, B7-2 and B7-3, or another co-stimulatory cell-surface glycoprotein. Preferably the co-stimulatory domain is a portion of B7-1 or B7-2, more preferably the complete extracellular portion of B7-1 or B7-2.

The SCM is formed by expression of a novel gene encoding a fusion protein containing the antigen-binding domain or domains and the co-stimulatory domain or domains. If the antigen-binding domain is comprised of a heavy and a light chain, the co-stimulatory domain is fused to one or other of the immunoglobulin chains, preferably to the heavy chain. If the antigen-binding domain is a scFv, the co-stimulatory domain is fused to the scFv. The domains can be placed in the order (N-terminus to C-terminus): antigen-binding domain followed by co-stimulatory domain; or co-stimulatory domain followed by antigen-binding domain. Preferably, the co-stimulatory domain is placed at the N-terminus followed by the antigen-binding domain. A signal peptide is included at the N-terminus, and may be for example the natural signal peptide of the co-stimulatory extracellular domain. The different domains may be separated by additional sequences, which may result from the inclusion of convenient restriction-enzyme cleavage sites in the novel gene to facilitate its construction, or serve as a peptide spacer between the domains, or serve as a flexible peptide linker or provide another function. Preferably the domains are separated by a flexible linker.

Two or more different genes encoding different SCMs may be used to achieve improved co-stimulation, or both co-stimulation of naïve T-cells and induction of memory responses. For example a gene encoding an SCM containing the B7-1 extracellular domain may be administered with a gene encoding an SCM containing the B7-2 extracellular domain.

Thus in one aspect of the invention, there is provided one or more genetic vectors capable of expressing in mammalian cells one or

more secreted co-stimulatory molecules, each secreted co-stimulatory molecule comprising at least one antigen-binding domain and at least one domain from the extracellular portion of a cell-surface co-stimulatory molecule. The co-stimulatory domain may be obtained from a molecule expressed on the surface of an antigen-presenting cell such as a B7 family member. Preferably the co-stimulatory domain is from B7-1, B7-2 or B7-3. Most preferably it is comprised of B7-1 amino acid residues 1 to approximately 215 of the mature B7-1 molecule (described in WO96/00092) or amino acids 1 to approximately 225 of the mature cell-surface form of B7-2 (described in Gerstmeyer et al. 1997 J. Immunol. 158:4584-4590).

The genetic vector according to this aspect of the invention comprises at least a promoter and enhancer for expression in mammalian cells and a polyadenylation site. Suitable promoters and enhancers include the MIE promoter-enhancer from human cytomegalovirus or promoters which are expressed preferentially in cells present within the tumour. Such promoter-enhancers include those from the MUC1 gene , the CEA gene or the 5T4antigen gene. If two or more SCMs are expressed, the coding regions for these may be inserted into two separate vectors or a single vector may be used to express the two or more genes. In the latter case each gene is provided with a separate copy of the promoter, or an internal ribosome entry site (IRES) is used to separate the two coding sequences.

In a second aspect of the invention, there is provided a gene delivery system for targeting one or more genes encoding SCMs of the first aspect of the invention to a tumour. The gene delivery system of this aspect of the invention comprises a genetic vector encoding a SCM and an *in vivo* gene-delivery system. The gene delivery system may be a non-viral gene delivery system such as DNA compacted with a DNA-compaction agent, or a liposome or immunoliposome which may contain

DNA compacted with a DNA-compaction agent such as poly-lysine. The vector may be a plasmid DNA vector. Alternatively the vector may be a recombinant viral vector such as an adenovirus vector, an adeno-associated virus (AAV) vector, a herpes-virus vector or a retroviral vector in which case gene delivery is mediated by viral infection of a target cell. Preferably the vector is a recombinant retroviral vector, which may be a targeted retroviral vector. Preferably, the retroviral vector is resistant to human complement, for example by production in a human cell line. In any event, the vector will contain a promoter to direct expression of the or each therapeutic gene and may contain additional genetic elements for the efficient or regulated expression of SCM genes, including enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals. The promoter and/or enhancer may be tissue-restricted in its activity. For example a tumour-specific promoter-enhancer, such as a 5T4 antigen gene promoter-enhancer or the CEA-gene promoter-enhancer may be used. Alternatively or additionally, an element or elements for regulated expression may be present, such as a hypoxia regulated enhancer. An example of a hypoxia regulated expression element (HRE) is a binding element for the transcription factor HIF1. Preferably expression of the or a therapeutic gene is inducible by hypoxia (or low oxygen supply) such as may be found in a tumour mass. Most preferably, the promoter and/or enhancer directing expression of the therapeutic gene contains both hypoxia-responsive elements and elements which give higher expression in tumour cells than in neighbouring non-tumour cells.

Additional vector components will be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication, and integration as appropriate using components which are well known in the art.

In a preferred embodiment of this aspect of the invention, a

retroviral vector is used for *in vivo* delivery of the genes encoding the SCM or SCMs to the tumour. Suitable retroviral vectors are known in the art (see for example Gunzberg and Salmons 1996 In Gene Therapy ed. Lemoine and Cooper. Bios; and Cosset et al. 1995 J. Virol. 69; 7430-7436). In a particularly preferred embodiment, expression of the SCM may be enhanced in the hypoxic regions of the tumour by the inclusion of hypoxia regulated genetic elements in the retroviral vector. In this case, the hypoxia-regulated elements may be inserted into one or both of the retroviral LTRs in place of the LTR enhancer or in another position in the vector by standard molecular biology techniques. The gene or genes encoding the SCM may be expressed from a promoter-enhancer which leads to enhanced expression in the tumour cells compared with neighbouring non-tumour cells or is preferably essentially tumour-specific. Examples of suitable promoters include the promoter-enhancer of the gene for 5T4 antigen, or the promoter-enhancer of the MUC1 gene or a CEA gene.

In a third aspect of the invention there is provided a method of treating cancer in a human or non-human mammal, comprising administering the SCM gene or genes in a gene delivery system of the first aspect of the invention either systemically or directly to the site of a tumour.

In a fourth aspect of the invention, is provided a gene delivery system for introducing one or more genes encoding one or more SCMs into cells of the myeloid haematopoietic cell lineage either *in vivo* or *ex vivo*. Preferably the myeloid cells are of the monocyte-macrophage lineage. Alternatively, the cells are precursor cells which have the capacity to differentiate into monocytic cells, such as CD34-positive haematopoietic stem cells. For *ex vivo* delivery, the genes can be inserted into a plasmid vector and delivered by one of a variety of DNA transfection methods including electroporation, DNA biolistics, lipofection or compacted DNA-mediated transfection. Alternatively a viral vector can be used to

transduce myeloid cells or stem cells *ex vivo,* such as an adenovirus vector, a retroviral vector or a lentiviral vector. The vector will contain a promoter to direct expression of the or each therapeutic gene and may contain additional genetic elements for the efficient or regulated expression including enhancers, translation initiation signals, internal ribosome entry sites (IRES), splicing and polyadenylation signals. The promoter or an enhancer or splicing signals may be tissue-restricted and preferentially active in mononuclear phagocytes such as macrophages. The promoter and/or enhancer may contain elements for regulated expression such as a hypoxia-regulated enhancer. An example of a hypoxia regulated expression element is HIF1 transcription factor response element. Such an element may be present in multiple copies. Examples of hypoxia-regulated promoters and enhancers include those from the enolase gene, the erythropoietin gene, and genes encoding glycolytic enzymes (Semenza et al., 1994 J. Biol. Chem 269; 23757-23763) such as the PGK gene. Isolated HREs can be multimerised in order to increase the response to hypoxia.

Additional vector components may be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication and integration as appropriate.

After introduction of the vector into the cells *ex vivo*, the cells can be re-introduced into the patient directly or they can be stimulated to differentiate along the monocyte-macrophage differentiation pathway using appropriate combinations of cytokines and growth factors prior to re-introduction into the patient. CD34-positive cells are stimulated to differentiate using cytokines including IL-3, GMCSF and MCSF. Monocytes are differentiated either by culture attached to plastic or using GMCSF either alone or in combination with other cytokines including MCSF.

For introduction of therapeutic genes into myeloid cells or

CD-34 positive stem-cells *in vivo*, a suitable *in vivo* delivery system can be used to deliver the transcription units described above. The gene delivery system may be a non-viral gene delivery system such as DNA compacted with a DNA-compaction agent, or a liposome or immunoliposome which may contain DNA compacted with a DNA-compaction agent. Alternatively the vector may be a recombinant viral vector such as a targeted adenovirus vector, an adeno-associated viral (AAV) vector, a herpes-virus vector or a retroviral vector such as a lentiviral vector. Preferably the vector is a targeted recombinant lentiviral vector, which is preferably resistant to human complement, for example by preparation of the vector from a human packaging cell line.

CD34-positive stem cells can also differentiate to form endothelial cells (Ashara et al. 1997 Science 275; 964-967). Such a route of differentiation for CD34 positive stem cells containing SCM encoding genes according to the invention is envisaged in addition to differentiation to form monocytes and macrophages.

Additional vector components may be provided for other aspects of vector function such as vector maintenance, nuclear localisation, replication, and integration as appropriate using components which are well known in the art.

In a preferred embodiment of this aspect of the invention, a plasmid vector or a retroviral vector carrying a gene encoding a SCM under the control of a hypoxia regulated promoter or a promoter active in macrophages is introduced into autologous peripheral blood monocytes. The transfected monocytes are re-introduced into the patient where they migrate to the hypoxic regions of tumours permitting enhanced production of the SCM in the interior of the tumour mass. The macrophages are optionally treated with cytokines prior to re-injection into the patient. Alternatively or additionally the vector may include DNA sequences capable of expressing a cytokine gene such as a gene for IFNg, CSF-1 or

GM-CSF in order to elicit the differentiation of the transfected cells. The cytokine gene may also be regulated by genetic elements which show enhanced activity at the site of the tumour.

In a fifth aspect of the invention, there is provided a method for treating cancer in a human or non-human mammal, comprising withdrawing an amount of blood from an individual suffering from cancer, preparing a cell preparation enriched in monocytes and macrophages or their stem-cell progenitors, introducing SCM genes into the cell preparation using a gene delivery system of the second aspect of the invention so as to bring about transfection or transduction of the monocytes and macrophages, or their stem-cell progenitors with the SCM genes, and re-introducing the transfected or transduced cells either systemically or directly to the site of the tumour. The cell preparations may optionally be treated with cytokines prior to re-introduction in order to elicit differentiation towards active macrophages.

In a further aspect of the invention is provided a method for treating cancer in a mammal, comprising administering to an individual a gene delivery system of the fourth aspect of the invention capable of expressing one or more SCMs in cells derived from a myeloid origin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequence of 5T4scFv.1

Figure 2 shows the sequence of B7-1.5T4.1

Figure 3 shows a diagrammatic representation of two SCMs based on the B7-1 co-stimulatory domain; Figure 3a shows the SCM B7-1.5T4.1 and Figure 3b shows B7-1.5T4.2 in which the order of the co-stimulatory and tumour-binding domains are reversed. Sp = signal peptide; B7 ec = extracellular domain of B7-1; Vl = light chain variable domain of 5T4; Vh = heavy chain variable domain of 5T4.

Figure 4 shows the sequence of the extracellular domain of

human B7-2, including the signal peptide sequence. The mature protein begins at amino acid 17. The B7-2 derived sequence is followed by a flexible linker gly-gly-gly-gly-ser.

DETAILED DESCRIPTION

In accordance with the invention, standard molecular biology techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook et al. (1989) Molecular Cloning; a laboratory manual; Hames and Glover (1985 – 1997) DNA Cloning: a practical approach, Volumes I-IV (second edition). Methods for the engineering of immunoglobulin genes in particular are given in McCafferty et al (1996) Antibody engineering: a practical approach.

A genetic "vector" is a genetic element (such as a plasmid, chromosome, artificial chromosome or a virus) which functions to transfer a segment of heterologous DNA into a target cell. Once within the target cell, the vector may serve to maintain the heterologous DNA within the cell or may act as a unit of DNA replication.

A "promoter" is a DNA sequence capable of binding an RNA polymerase enzyme within a cell and directing the initiation of transcription of a coding sequence at a specific site on the DNA.

An "enhancer" is a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

An "intron" is an intervening sequence of DNA within a gene which is removed by RNA splicing and so is not present in the mature messenger RNA and does not code for protein. Introns can be conditional or alternatively spliced in different cell types.

A "retrovirus" is a virus which contains genomic RNA which on entry into a host cell is converted to a DNA molecule by a reverse

transcriptase enzyme. The DNA copy serves as a template for the production of new RNA genomes and virally encoded proteins necessary for the assembly of infectious viral particles. The term "recombinant retroviral vector" describes a DNA molecule which contains sufficient retroviral sequences to allow an RNA transcript of the vector to be packaged in the presence of essential retroviral proteins into a retroviral particle capable of infecting a target cell. Infection of the target cell includes reverse transcription and integration into the target cell genome. The term "recombinant retroviral vector" also covers a retroviral particle containing an RNA genome encoded by the DNA molecule. The retroviral vector will also contain non-viral genes which are to be delivered by the vector to the target cell. A recombinant retroviral vector is incapable of independent replication to produce infectious retroviral particles. Usually, a recombinant retroviral vector lacks functional *gag-pol* and/or *env* genes, or other genes encoding proteins essential for replication. A "targeted retroviral vector" is a recombinant retroviral vector whose ability to infect a cell or to be expressed in the target cell is restricted to certain cell types within the host organism. An example of targeted retroviral vectors is one with a genetically modified envelope protein which binds to cell surface molecules found only on a limited number of cell types in the host organism. Another example of a targeted retroviral vector is one which contains promoter and/or enhancer elements which permit expression of one or more retroviral transcripts in only a proportion of the cell types of the host organism.

An "envelope" protein is a viral protein which coats the viral particle and plays an essential role in permitting viral entry into a target cell.

"Transduction" is the process of using a viral vector to deliver a non-viral gene to a target cell. "Transfection" is a process using a non-viral vector to deliver a gene to a target mammalian cell.

"Immunoglobulin" is the term given to a member of a family of

related multimeric proteins which are normally secreted from cells of the B-lymphocyte lineage of a vertebrate, whose function is to bind specifically with a region of a macromolecule identified as non-self. Immunoglobulins represent a major component of the immune response repertoire of the organism and are synonymous with "antibodies".

The invention relates to the production of novel co-stimulatory fusion molecules from mammalian cells and delivery of SCM-encoding genes to the site of a tumour. Delivery of SCM-encoding genes to the tumour has considerable advantages for therapeutic applications in which SCMs are indicated since it circumvents a number of problems associated with delivery of proteins systemically in man. Proteins are complex molecules which, of necessity, are produced from biological sources, most usually from genetically engineered organisms or cell cultures. The procedures for production of SCMs are consequently complicated, labour intensive and costly. Furthermore, pharmacological properties and other aspects of the function of complex proteins such as SCMs, derived from non-human biological sources may frequently differ in important ways from the activity of the corresponding proteins produced in human cells. One major cause of such differences in activity is variations in the pattern of glycosylation of proteins derived from different species (reviewed in Bebbington 1995; In Monoclonal Antibodies: the second generation ed . H. Zola pg 165-181). Systemic administration of SCMs containing toxin domains can identify additional pharmacokinetic and toxicological problems (reviewed in Scheinberg and Chapman 1995. In Monoclonal antibodies (ed. Birch and Lennox) Chapter 2.1).

In contrast to the problems associated with production and delivery of proteins, the methods of the invention allow the delivery of genes to the site of the tumour, thus circumventing a number of production problems. The SCMs are thereby produced *in situ* in the autologous human cells, which serve as a local factory for the production of the gene-

based therapeutic. This has significant advantages in minimising systemic toxicity. The activity of the protein is maximal since the glycosylation of the protein shows a human pattern appropriate to the individual being treated.

The methods of the invention can be used in conjunction with direct injection into the site of the tumour or systemic delivery of, for example targeted vectors or engineered myeloid cells or their progenitors. Systemic delivery may be particularly advantageous in a number of indications, particularly in the treatment of disseminated disease. In these cases the gene delivery system or engineered cells can be administered intravenously by bolus injection or by infusion in a suitable formulation. A pharmaceutically acceptable formulation may include an isotonic saline solution, a buffered saline solution or a tissue-culture medium. Additional formulatory agents may be included such as preservative or stabilising agents.

The invention will now be further described by way of examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1 - Construction of B7 – scFv Fusion proteins

The extracellular domain of B7-1 is defined by amino-acid residues 1 – 215 of the native human B7-1 protein. This sequence, together with its signal peptide-encoding sequence, is used to construct secreted fusion proteins which also contain the scFv derived from the 5T4 monoclonal antibody. The sequence of the 5T4 scFv is given in Figure 1.

A DNA coding sequence is constructed using standard molecular biology techniques which encodes a fusion protein in which the

N-terminus of the 5T4 scFv is fused after amino acid 215 of human B7-1. The sequence of this coding sequence, B7-1.5T4.1, is shown in Figure 2. The fusion protein contains a flexible (gly-gly-gly-gly-ser) spacer between the B7-1 and 5T4 scFv sequences. The introduction of a convenient BamH1 restriction site at the end of the linker insertion (beginning at nucleotide 733) also allows for further linkers to be screened for optimal expression of bi-functional fusion protein. Figure 3 indicates the fusion protein in diagrammatic form. It is similarly possible to construct B7-1.5T4.2 (Figure 3b) in which the scFv is N-terminal and the B7 extracellular domain is C-terminal. In this case only the coding sequence of the mature B7-1 (without signal peptide) is required. A signal peptide such as an immunoglobulin leader sequence is added to the N-terminus of the scFv in this instance.

For fusion proteins which use the co-stimulatory extracellular domain of B7-2, the signal peptide and extracellular domain of B7-2 is used in place of B7-1 sequences. Figure 4 shows the coding sequence of the SCM B7-2.5T4.1co-stimulatory domain. It encodes the first 225 amino acids of human B7-2, preceded by its signal peptide, and a flexible linker (gly4-ser). The BamHI site at the end of this sequence can be used to insert the domain upstream of the 5T4scFv.1 (see Figure 3). The sequence includes the B7-2 signal peptide which can serve to allow secretion of this fusion protein in which the B7-2 domain is at the N-terminus of the fusion protein.

Each engineered cDNA is inserted into the mammalian expression vector pCI to allow expression in mammalian tissue culture cells. For this purpose, a linker sequence is added to the 5'-end of the coding sequence which introduces a convenient restriction site for insertion into the polylinker of pCI and adds the translation initiation signal CCACC immediately adjacent to the first ATG codon. Constructs in pCI are transfected into a suitable mammalian host cell line such as COS-1 to

confirm secretion of the SCM. The transcription cassette from pCI or an appropriate segment of the transcription cassette is subsequently sub-cloned into the expression vector to be used as the gene delivery system for therapeutic use.

Example 2 - Transfection of macrophages / monocytes with an expression vector encoding an SCM.

Peripheral blood mononuclear cells are isolated from human peripheral blood at laboratory scale by standard techniques procedures (Sandlie and Michaelsen 1996 In Antibody engineering: a practical approach. Ed McCafferty et al. Chapter 9) and at large scale by elutriation (eg Ceprate from CellPro). Adherent cells (essentially monocytes) are enriched by adherence to plastic overnight and cells can be allowed to differentiate along the macrophage differentiation pathway by culturing adherent cells for 1 – 3 weeks.

Monocytes and macrophages are transfected with an expression vector capable of expressing SCM in human cells. For constitutive high level expression, the SCM is expressed in a vector which utilises the hCMV-MIE promoter-enhancer, pCI (Promega). For hypoxia-induced expression, the hCMV promoter is replaced by a promoter containing at least one HRE. A suitable promoter is a truncated HSV TK promoter with 3 copies of the mouse PGK HRE (Firth et al. 1994 Proc. Natl. Acad. Sci. 91: 6496-6500).

A variety of transfection methods can be used to introduce vectors into monocytes and macrophages, including particle-mediated DNA delivery (biolistics), electroporation, cationic agent-mediated transfection (eg using Superfect, Qiagen). Each of these methods is carried out according to the manufacturer's instructions, taking into account the parameters to be varied to achieve optimal results as specified by the

individual manufacturer. Alternatively, viral vectors may be used such as defective Adenovirus vectors (Microbix Inc or Quantum Biotechnologies Inc).

Example 3 - Analysis of SCM binding to CTLA-4 and 5T4-antigen expressing cells

The B7-1 or B7-2 domains are expected to bind specifically to CD28 and CTLA-4 present on human T-cells. Binding to T-cells or Chinese hamster ovary cells transfected with human CTLA-4 or CD28 is determined using FACS analysis as follows. 5 x 10^5 CTLA-4 expressing target cells or equivalent cells lacking CTLA-4 (untransfected CHO cells) are incubated with 0.1 ml culture supernatant from COS-1 cells transiently transfected with SCM genes for 1 h at 4oC. The cells are washed and incubate with 1 mg monoclonal antibody specific for the B7 domain (eg Mab 9E10) followed by FITC-labelled goat anti-mouse IgG (Pharmingen) and analysis by FACS.

Binding of scFv to 5T4-antigen is similarly assessed using target cells expressing 5T4-antigen (5T4-transfected A9 cells) or control cells (A9).

Example 4 - Analysis of co-stimulatory activity

An established mouse cell line of Balb/c origin such as HC11 cells is transfected with the cDNA encoding human 5T4-antigen (Myers et al. 1994 J. Biol. Chem. 269; 9319-9324) inserted in the expression vector pCIneo.

Splenic T-cells from Balb/c mice are isolated by standard procedures (Johnstone and Thorpe 1996 In Immunochemistry in Practice. Blackwell. Chapter 4). T-cells are pre-stimulated by incubation for 1 – 2

days in medium containing 10ng/ml PMA (Sigma) and 100 U/ml human IL-2 (Boehringer Mannheim). HC11-5T4 cells are incubated at 10^4 cells /well of a 96-well tissue culture tray for 2 h with up to 0.1ml supernatant from COS cells transfected with SCM gene. Up to 10^5 pre-stimulated T-cells are added to each well, the cells are pulsed with 0.25 mCi / well ^{3}H-thymidine and incorporation of ^{3}H-thymidine is measured using a liquid scintillation counter after 24h.

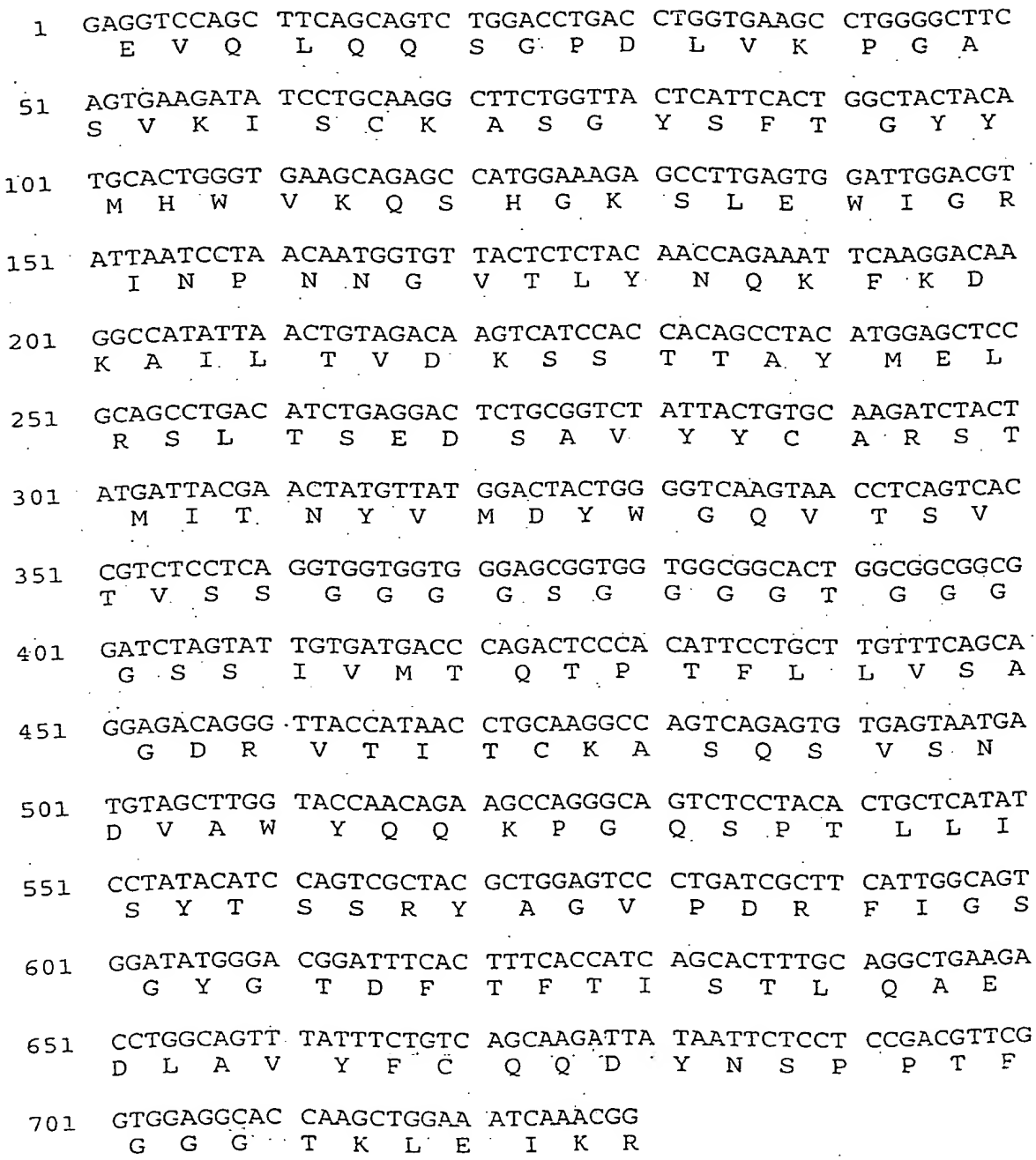
Incorporation of ^{3}H-thymidine is anticipated to be enhanced by the presence of SCM.

Example 5 - Analysis of co-stimulation in animal models.

HC11 cells transfected with the human 5T4-antigen gene (Example 4) are grown as tumours in Balb/c mice. SCM genes B7-1.5T4.1 or B7-2.5T4.1 or a combination of both genes are introduced into the tumour cells prior to implantation and the growth of the tumours and the growth of control tumours which do not express SCM genes *in vivo* are monitored.

It is anticipated that the expression of SCM genes will lead to significant reduction in tumour growth.

Figure 1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E V Q L Q Q S G P D L V K P G A
51 AGTGAAGATA TCCTGCAAGG CTTCTGGTTA CTCATTCACT GGCTACTACA
S V K I S C K A S G Y S F T G Y Y
101 TGCACTGGGT GAAGCAGAGC CATGGAAAGA GCCTTGAGTG GATTGGACGT
M H W V K Q S H G K S L E W I G R
151 ATTAATCCTA ACAATGGTGT TACTCTCTAC AACCAGAAAT TCAAGGACAA
I N P N N G V T L Y N Q K F K D
201 GGCCATATTA ACTGTAGACA AGTCATCCAC CACAGCCTAC ATGGAGCTCC
K A I L T V D K S S T T A Y M E L
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T V S S G G G G S G G G G T G G G
401 GATCTAGTAT TGTGATGACC CAGACTCCCA CATTCCTGCT TGTTTCAGCA
G S S I V M T Q T P T F L L V S A
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G D R V T I T C K A S Q S V S N
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D V A W Y Q Q K P G Q S P T L L I
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S Y T S S R Y A G V P D R F I G S
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G Y G T D F T F T I S T L Q A E
651 CCTGGCAGTT TATTTCTGTC AGCAAGATTA TAATTCTCCT CCGACGTTCG
D L A V Y F C Q Q D Y N S P P T F
701 GTGGAGGCAC CAAGCTGGAA ATCAAACGG
G G G T K L E I K R

gure 2

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+1 Gly His Asn Val Ser Val Glu Glu Leu Ala Gln Thr Arg Ile Tyr Trp Gln

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+1 Ile Val Ile Leu Ala Leu Arg Pro Ser Asp Glu Gly Thr Tyr Glu Cys Val

ATTGTGATCC TGGCTCTGCG CCCATCTGAC GAGGGCACAT ACGAGTGTGT 350
TAACACTAGG ACCGAGACGC GGGTAGACTG CTCCCGTGTA TGCTCACACA

+1 - Val Leu Lys Tyr Glu Lys Asp Ala Phe Lys Arg Glu His Leu Ala Glu Val

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Figure 2 Cont.

```
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gure 2 Cont.

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Figure 3a
B7-1.5T4.1

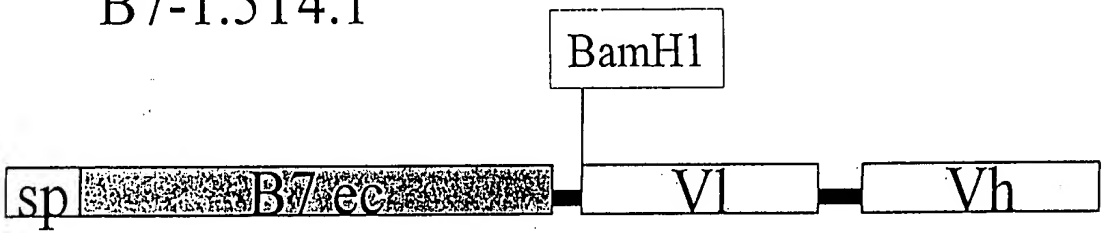

Figure 3b
B7-1.5T4.2

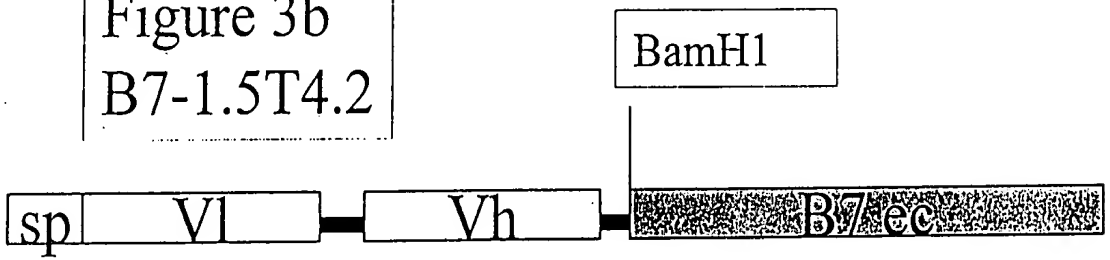

Figure 4

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Description:

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 201  GAAATTTGAC AGTGTTCATT CCAAGTATAT GGGCCGCACA AGTTTTGATT
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